SUPPLEMENTARY APPENDIX

Platelet HIF-2α promotes thrombogenicity through PAI-1 synthesis and extracellular vesicle release

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Supplemental data

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Platelet HIF-2α induces prothrombotic state

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Supplemental Data includes:

Legends to Supplemental Videos 1-3 and Supplemental Videos (accelerated motion, 16X)

Methods

Figures: Supplemental Figure 1 and 2

Table: Supplemental Table 1

Legends to Supplemental Videos 1-3

Supplemental Video 1. Ferric chloride-induced mesenteric arteriolar thrombosis in mice pre-administered with DMOG. Platelets were fluorescently labeled with DyLight 488 anti-GPIbβ antibody (0.1 μg/g body weight).

Supplemental Video 2. Ferric chloride-induced mesenteric arteriolar thrombosis in mice pre-administered with DFO. Platelets were fluorescently labeled with DyLight 488 anti-GPIbβ antibody (0.1 μg/g body weight).

Supplemental Video 3. Ferric chloride-induced mesenteric arteriolar thrombosis in mice pre-administered with vehicle (control). Platelets were fluorescently labeled with DyLight 488 anti-GPIbβ antibody (0.1 μg/g body weight).

Legend to Supplemental Videos 1-3 (accelerated motion, 16X)

Supplemental Video 1 (accelerated motion, 16X). Ferric chloride-induced mesenteric arteriolar thrombosis in mice pre-administered with DMOG. Platelets were fluorescently labeled with DyLight 488 anti-GPIbβ antibody (0.1 μg/g body weight).

Supplemental Video 2 (accelerated motion, 16X). Ferric chloride-induced mesenteric arteriolar thrombosis in mice pre-administered with DFO. Platelets were fluorescently labeled with DyLight 488 anti-GPIbβ antibody (0.1 μg/g body weight).

Supplemental Video 3 (accelerated motion, 16X). Ferric chloride-induced mesenteric arteriolar thrombosis in mice pre-administered with vehicle (control). Platelets were fluorescently labeled with DyLight 488 anti-GPIbβ antibody (0.1 μg/g body weight).

Methods

Materials

Antibodies against HIF-1α (ab463) and HIF-2α (NB100-122) were procured from Abcam and Novus Biologicals, respectively. Anti-actin antibody (A2066), acetylsalicylic acid, ethylene glycol tetra-acetic acid (EGTA), ethylene di-amine tetra-acetic acid (EDTA), sodium orthovanadate, skimmed milk powder, thrombin, bafilomycin A1, chloroquine, 3-methyladenine, xylazine, DMOG, DFO and DMSO were obtained from Sigma. Anti-PAI-1 antibody (IM29L), proteasome inhibitors (PS I and MG 132) and Fura-2 AM were purchased from Calbiochem. ADP and collagen were procured from Chrono-log. Polyvinylidene fluoride (PVDF) membrane and enhanced chemiluminescence (ECL) kit were from Millipore, TRIzol from Invitrogen, and diethylpyrocarbonate (DEPC) and bovine serum albumin were from Amresco. Ketamine was from Neon Laboratories Ltd and DyLight 488-labled anti-GPIbβ antibody (X488) was from Emfret Analytics. High-capacity reverse transcription kit was purchased from Applied Biosystems. Primers (forward and reverse) were from Eurofins Genomics and SYBR Green Supermix (2X) was obtained from Bio-Rad. Horseradish peroxidase (HRP) –conjugated secondary antibodies were procured from Bangalore Genei. Reagents used in electrophoresis were from Merck. All other reagents were of analytical grade. Type I deionized water (18.2 MΩ.cm, Millipore) has been used throughout the experiments.

Platelet preparation

Fresh venous blood was collected in citrated-phosphate-dextrose-adenine (citric acid anhydrous, 15 mM; sodium citrate dehydrate, 86 mM; monobasic sodium phosphate, 16

mM; and dextrose, 130 mM) under informed consent as per recommendations by the Institutional Ethical Committee. Briefly, blood was centrifuged at 180×g for 10 min. Platelet-rich plasma (PRP) thus obtained was incubated with 1 mM acetylsalicylic acid for 15 min at 37 ˚C. After addition of EDTA (5 mM), platelets were sedimented by centrifugation at 600 × g for 10 min. Cells were washed in buffer A (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4 and 1 mM EGTA, supplemented with 5 mM glucose). Platelets were finally resuspended in buffer B (pH 7.4), which was same as buffer A, but without EGTA. Final cell count was adjusted to 2.5-5.0×10^8 cells/ml. All steps were carried out under sterile conditions at room temperature and precautions were taken to maintain cells in resting state.

Western analysis

Platelet proteins were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and electrophoretically transferred onto PVDF membranes in a TE77 PWR semi dry blotter (GE Healthcare) at 0.8 mA/cm^2 (for 2 h in case of HIF-1α and -2α, and 1 h in case of PAI-1) or in a Trans-Blot Turbo Transfer System (Bio-Rad) at 20 V/1.3 A (for 30 min in case of HIF-2α, and 20 min in case of PAI-1). Membranes were blocked either with 5% skimmed milk and 1% BSA (for HIF-1α, -2α), or with 5% BSA (for PAI-1) in TBST (10 mM Tris HCl, 150 mM NaCl, pH 8.0 containing 0.05 % Tween 20) for 1 to 1.5 h at room temperature to block residual protein binding sites. Membranes were incubated overnight at 4 ˚C with primary antibodies (anti-HIF-1α, 1:500; anti-HIF-2α; 1:500; anti-PAI-1, 1:100; anti-actin, 1:5000), followed by 3 washings with TBST for 5 min each. Blots were incubated with HRP-conjugated secondary antibodies (goat anti-mouse, 1:1500, for HIF-1α and PAI-1; goat anti-rabbit, 1:2000, for HIF-2α, and 1:40000, for actin) for 1 h at RT, followed

by washing. Antibody binding was detected using enhanced chemiluminescence. Images were acquired on multispectral imaging system (UVP BioSpectrum 800 Imaging System) and quantified using VisionWorks LS software (UVP). β-actin was used as protein loading control.

Total RNA extraction and reverse transcription

(a) RNA extraction

Platelets isolated from fresh human blood were counted in Beckman Coulter Multisizer 4 to adjust to final count at 4-8×10^8 cells/ml. Precautions were taken to preclude leukocyte contamination. Total RNA was extracted from platelets by using TRIzol reagent (containing guanidinium thiocyanate and phenol) according to manufacturer's protocol and suspended in DEPC-treated RNase-free water. RNA concentrations were determined at 260/280 nm in multimodal microplate reader (BioTek model Synergy H1).

(b) Reverse transcription

RNA (1 μg) isolated from human platelets was reverse transcribed to complementary DNA (cDNA) by using high-capacity cDNA reverse transcription kit (Applied Biosystems) according to manufacturer's protocol, in a PTC-150 thermal cycler (MJ Research). The optimized thermal cycling conditions were 25 ˚C for 10 min, 37 ˚C for 2 h, 85 ˚C for 5 min. The cDNA obtained were stored at 4 ˚C.

Quantitative real-time polymerase chain reaction

Following were the primer sequences designed using latest version of Primer3 Input software: (I) HIF-1α - ATGTAATGCTCC CCTCACCC (F), CAGGGTCAGCACTACTTCGA (R) (II) HIF-2α - TCGGAGAGGAGGAAGGAGAA (F), GAGGAGAGGAGCTTGTGTGT (R). Glyceraldehyde-3-phosphate dehydrogenase **(**GAPDH) gene was used as the reference control and the primer

sequences for GAPDH gene were GAAGGTGAAGGTCGGAGTC (F) and GAAGATGGTGATGGGATTTC (R).

The qPCR was carried out in a CFX-96 real-time thermal cycler (Bio-Rad) using SYBR Green SuperMix according to manufacturer's protocol. Expression of mRNA was considered significant when the critical quantity (Cq) value was less than 33. Amplifying thermal cycling conditions were 95 ˚C for 3 min initially, followed by 40 cycles consisting of denaturation for 10 sec at 95 ˚C, annealing for 10 sec (at 56 ˚C for GAPDH, and 59.2 ˚C for both HIF-1α and -2α) and extension at 72 °C. Melt peak analysis was performed to rule out presence of non-specific amplification.

Isolation and analysis of platelet-derived extracellular vesicles (PEVs)

Platelets were pre-treated either with hypoxia-mimetics (DMOG and DFO) or exposed to hypoxia for 2 h at room temperature. Cells were sedimented at 800×g for 10 min followed by 1200×g for 2 min at 22 ˚C to obtain PEVs cleared of platelets. PEVs in supernatant were analysed by Nanoparticle Tracking Analyzer (NTA). A beam from solid-state laser (638 nm) was allowed to pass through the sample. Light scattered by rapidly moving particles in suspension in Brownian motion at room temperature was observed under 20X microscope. This revealed hydrodynamic diameters of particles, calculated using Stokes Einstein equation, within range of 10 nm to 1 µm and concentration between 10^7–10^9/ml. The average distance moved by each PEV in x and y directions were captured by CCD camera (30 frames per sec) attached to the microscope. Both capture and analysis were performed using NanoSight LM10 (Malvern) and NTA 2.3 analytical software, which provide an estimate of the particle size and concentration of EVs in sample.

Intravital Imaging of mesenteric arteriolar thrombosis

The study was approved by the Central Animal Ethical Committee of Institute of Medical Sciences, Banaras Hindu University. All efforts were made to minimize the number of animals used, and their suffering. Mice (Swiss albino, 5-6 weeks old, 8-10 g each) were anaesthetized with intraperitoneal injection of ketamine/xylazine cocktail (100 mg/kg ketamine and 10 mg/kg xylazine). DyLight 488-labled anti-GPIbβ antibody (0.1 µg/g body weight) diluted in 50 µl sterile PBS was injected into retro-orbital vein of mice to fluorescently label circulating platelets. Mesentery was exposed through a mid-line incision in the abdomen and kept moist by superfusion with warm (37° C) sterile PBS. An epifluorescence inverted video microscope (Nikon model Eclipse Ti-E) equipped with monochrome CCD cooled camera was employed to image isolated mesenteric arterioles of diameter 100-150 µm. The arteriole was injured by topically placing a whatman filter paper saturated with ferric chloride (20%) solution for 3 min and thrombosis in the injured vessel was monitored in real time for 40 min or until occlusion. Movies were subsequently analyzed with Nikon image analysis software (NIS Elements) to determine (a) the time required for formation of first thrombus (>20 µm in diameter), (b) time required for occlusion of the vessel i.e. time required after injury till stoppage of blood flow for 30 sec, and (c) thrombus growth rate i.e. growth of a thrombus (>30 µm diameter) followed over a period of 3 min. Fold increase was calculated by dividing diameter of thrombus at given time (n) by the diameter of the same thrombus at time (0). Time 0 was defined as the time point at which thrombus diameter first reached approximately 30 µm.

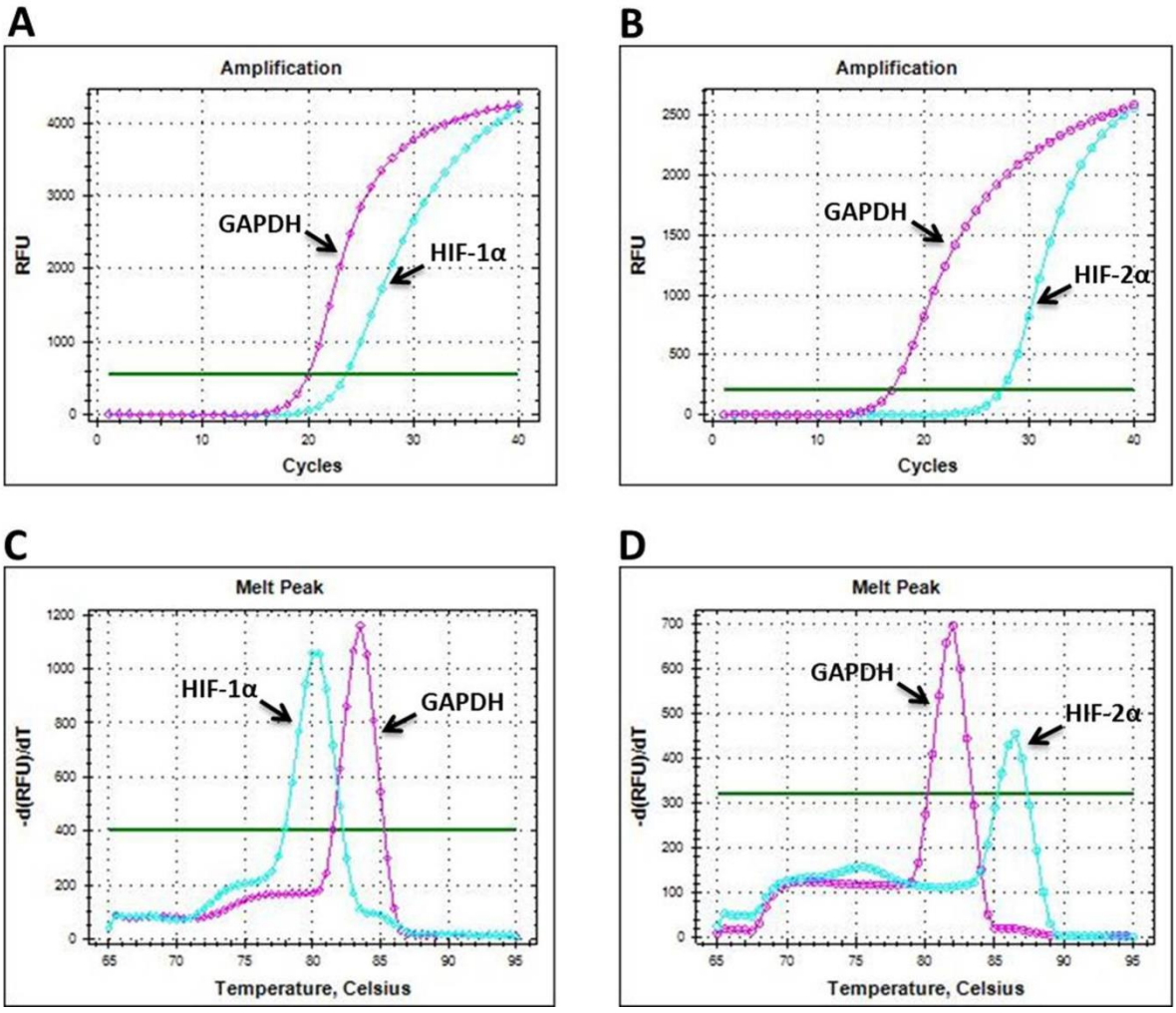
Measurement of intracellular free calcium

Platelet-rich plasma was incubated with Fura-2 AM (2 µM) at 37 ˚C for 45 min in dark. Fura-2 stained platelets were isolated, washed and finally resuspended in buffer B.

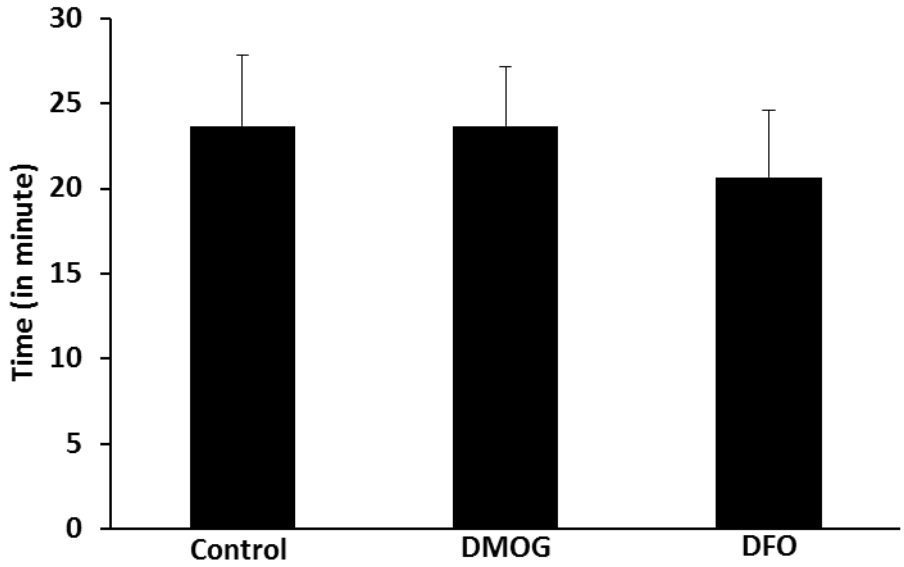
Fluorescence was recorded in 400 µl aliquots of platelet suspensions at 37 ˚C under non-stirring condition using Hitachi fluorescence spectrophotometer (model F-2500). Excitation wavelengths were 340 and 380 nm and emission wavelength was set at 510 nm. Changes in intracellular calcium concentration, $[Ca^{2+}]_{i,}$ was monitored from fluorescence ratio (340/380) using Intracellular Cation Measurement Program in FL Solutions software. F_{max} was determined by lysing the cells with 40 µM digitonin in presence of saturating $CaCl_2$. F_{min} was determined by the addition of 2 mM EGTA. Intracellular free calcium was calibrated according to the derivation of Grynkiewicz *et al.*

Statistics

Standard statistical methods were used in the study. Parametric methods (Student's *t* test) and Mann Whitney test were used for evaluation. Test was considered significant at $p<0.05$ (one-tailed tests). Data are represented as means ± SEM (standard error of mean) of at least three individuals from different blood donors.



Supplemental Figure 1. Human platelets abundantly express HIF-1α and HIF-2α genes at mRNA level. (A) and (B) Shows amplification chart of HIF-1α and HIF-2α genes at mRNA level in platelets after reverse transcription of 1 µg RNA to cDNA followed by qPCR. (C) and (D) Melt peak analyses to rule-out presence non-specific amplification. Figures are representative of five individual experiments.



Supplemental Figure 2. Bar diagram representing mean time to occlusion in mice pre-administered with vehicle (control), DMOG (400 mg/kg) or DFO (200 mg/kg) (n=3). Data are represented as mean ± SEM.

Supplemental Table 1. Arterial blood gas analysis of healthy controls and patients with COPD (analysed in pairs on the same day).

Days of analysis	Partial pressure of oxygen in arterial blood (PaO_2) (in mm Hg)	
	Healthy Controls	Patients with COPD
1	97.6	56.4
2	99.7	36.8
3	120	30.0
4	97	57.6
5	118	53.1
6	98	31.2
7	97	30.8
8	96	59.0
9	91.5	52.8
10	93	43.5